Experiments with Two Methods for the Study of Vitamin B

By

HARRIET ISABEL EDGEWORTH

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DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University, in the City of New York

NEW YORK CITY

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ACKNOWLEDGMENTS

The author is indebted to Professor H. C. Sherman for having suggested the problem and for advice and encouragement received throughout the work.

Thanks are also due to Professor A. W. Thomas for many helpful suggestions.

EXPERIMENTS WITH TWO METHODS FOR THE STUDY OF VITAMIN B

INTRODUCTORY

Investigations on the chemical nature of the vitamins by the usual analytical methods have not been successful, due to the fact that their instability has so far rendered their isolation impossible (1–10). However, a general method of procedure for studying these substances has been developed. This consists in feeding to a suitable organism, in an otherwise adequate diet, known amounts or proportions of a food supposed to contain the vitamin under investigation, as the sole source of this vitamin.

Three criteria or tests have been proposed for measuring the presence and relative amounts of the water-soluble B vitamin. These methods involve the use of the pigeon, the rat, and yeast cells as indicators of the vitamin present. The anti-neuritic content of a food has been tested for by its power to prevent or cure polyneuritis in pigeons or fowls. The water-soluble growth-promoting vitamin has been tested for by making the food to be studied the sole source of this vitamin in an otherwise adequate diet and (*a*) finding the amount necessary to induce normal growth in a young rat (11); or (*b*) determining the effect upon the growth of yeast in one of several ways (12–26). So far, it has not been fully demonstrated that any two of these methods necessarily measure the same thing. McCollum's view that the anti-neuritic and the growth-promoting "water-soluble B" vitamin are the same substance has been generally accepted and is tacitly assumed in the use of the term vitamin B to cover both. However, Mitchell, and Emmett and his co-workers have seriously questioned this view. Likewise some investigators have enthusiastically adopted the method of measuring in some way the accelerated growth of yeast as a test for this method. These workers believe that the experiments based on accelerated yeast growth serve the same purpose as those based on rat growth, and in a quicker, more readily controllable way, while other investigators have questioned whether the growth-promoting substance is the same in the two cases, and if so, whether the culture media used for the yeast are so adequate in all other respects as to make it safe to attribute the increased yeast growth solely to the vitamin.

OBJECT

The object of the experiments here reported was to investigate the most promising methods available for use in the quantitative study of vitamin B with respect to such problems as its stability toward heating.

SOURCE OF VITAMIN

Skimmed milk in the form of dry powder was chosen as the source of vitamin B because it is readily available, uniform, easy and accurate of manipulation in either large or small quantities, and furnishes "vitamin B" in a typical and natural form free from any danger of having been altered or fractioned by chemical or physical manipulation. (In case the so-called "vitamin B" is really a mixture of two substances, it was desired in these experiments that both should be present in the unchanged forms and proportion in which they exist in a typical complete food material such as milk.) It was also thought best, in view of the experience of this and other laboratories and of Osborne's suggestion that B may occur naturally in chemical combination with the proteins of some foods, to avoid the use of any method which should involve the assumption of a complete removal of vitamin B by means of solvents.

YEAST METHOD

Since Williams suggested the identity of the water-soluble B vitamin with the bios described by Wildiers, various methods have been proposed for measuring the vitamin content of a food by means of yeast stimulation (12–26). These include the measurement of the accelerated growth of yeast by (*a*) counting the cells produced; (*b*) determination of the carbon dioxide produced; (*c*) determination of the volume of cells produced; (*d*) weighing the cells produced (gravimetric).

Since the results obtained from counting the cells produced may vary as greatly as 5 and 123 in a control, and 24 and 9,724 in a solution containing vitamin (14), this method is not a satisfactory one from the standpoint of a quantitative discussion of results.

In the estimation of yeast growth by means of carbon dioxide production, there is the difficulty that the carbon dioxide in the solution depends on the pressure of carbon dioxide over the surface of the solution. Since this pressure is much greater than the partial pressure of carbon dioxide in the atmosphere there is a tendency for the carbon dioxide produced during the process of growth to pass through and out of the solution (27). Changes in temperature also affect this process. Since the loss from this source may be considerable, the method becomes one of doubtful value.

In determining the volume of cells produced, the fact that yeast is made up of protein having an isoelectric point on either side of which it will show differences in amount of swelling, depending on the hydrogen ion concentration of the medium, must be taken into account and hence reduces the ease and precision of this method.

The gravimetric method was adopted at the beginning of this work because it was believed to be the procedure giving the most constant and precise results from the standpoint of chemical analysis.

Yeast was made up for seeding from a Fleischmann cake according to the method of Williams (25). This was grown for eighteen hours at 30° C., in a constant temperature oven.

EXPERIMENTS WITH WILLIAMS MEDIUM

One hundred cc. of this medium contains:

Grams	
1.5	asparagine
0.25	$CaCl_2$
0.25	$MgSO_4$
2.00	KH_2PO_4
3.00	$(NH_4)2SO_4$
20.00	sugar

This medium was made up in 1,000 cc. portions and each of ten aliquot portions from this used as a test. The aliquot portions of medium in Erlenmeyer flasks, stoppered with cotton, were sterilized in an autoclave at 10 pounds pressure for fifteen minutes. When cooled to 30° C., 1 cc. of yeast suspension containing 0.3 mg. of yeast made according to Williams method was added to each portion. These were then incubated at 30° C. for eighteen hours. At the end of this period of incubation 1 cc. of U.S.P. formaldehyde was added to each flask to prevent further yeast growth, and the contents were subsequently poured into a weighed Gooch crucible prepared according to Williams (25), washed, dried at 102° C. for two hours, and weighed to constancy. This was considered a control as far as the yeast and medium were concerned, as previous experiments had shown that the medium itself contained only soluble material. Similar tests were made to which were added varying amounts of skimmed milk used subsequently as a source of vitamin B on rats, and modified by heating for six, twelve, twenty-four, and forty-eight hours respectively at 100° C. A second control consisting of the medium plus the amount of milk used as a source of vitamin was always carried out, and the weight of the two controls subtracted from the weight obtained in the test was considered as the amount of yeast growth due to the stimulating action of the vitamin. The concentration of hydrogen

ion in (*a*) the control, (*b*) the medium containing the yeast and 0.4 gm. of unheated milk, and (*c*) the medium containing the yeast and 0.4 gm. of milk heated forty-eight hours at 100° C. was as follows before and after incubation.

TABLE I

	P_H+ before Incubation	P_H+ after Incubation
Control+yeast	4.4	5.5
Control+yeast+0.4 gm. unheated milk	5.7	7.1
Control+yeast+0.4 gm. milk heated 48 hours	5.5	7.0

While the media seem to become slightly less acid during the process of incubation the change is not significant in this range, as far as the precipitation of the milk proteins are concerned (28, 29). Therefore the amount of milk precipitated, filtered off, and weighed with the yeast can be considered as the weight of the second control.

RESULTS OF EXPERIMENTS WITH WILLIAMS MEDIUM

The results of a typical set when 0.4 gm. of unheated milk powder was used are found in Table II.

TABLE II

	Media and Yeast (Gram)	Media and Milk (Gram)	Media, Milk, and Yeast (Gram)
	.0033	.1119	.1550
	.0029	.1076	.1557
	.0028	.1058	.1577
	.0031	.1094	.1548
	.0028	.1059	.1569
Av.	.0030	.1098	.1553
		.1117	.1547
		.1087	.1575
		Av. .1088	.1585
			.1601
			.1554
			.1544
			.1607
			.1617
			.1601
			Av. .1572

	Gram	
Average total weight	0.1572	
Average control weight	0.1118	(0.0030+0.1088)
Accelerated growth	0.0454	

TABLE III

COMPARISON OF RESULTS WITH UNHEATED AND HEATED MILK POWDER

Milk Powder Added	No. of Tests	Average Growth (Grams)	Probable Error of Mean (Grams)	Accelerated Growth (Grams)	Probable Error of the Difference (Grams)
None	60	.0031	.00004	.....	.....
0.1 gm.	17	.0174	.00027	.014	.00027
0.2 gm.	14	.0285	.00026	.026	.00026
0.4 gm.	39	.0482	.00025	.045	.00025
0.6 gm.	16	.0430	.00027	.040	.00027
0.4 gm. heated 12 hours at 100° C.	19	.0390	.00040	.036	.00040
0.4 gm. heated 24 hours at 100° C.	13	.0390	.00036	.036	.00035
0.4 gm. heated 48 hours at 100° C.	20	.0377	.00056	.034	.00056

DISCUSSION OF RESULTS

The results show an increase in yeast growth which was approximately proportional to the amount of unheated milk included up to 0.4 gm. per 100 cc. of medium. This agrees with the Williams findings when vitamin from other sources was included (25).

Since the maximum growth was obtained with 0.4 gm. of unheated milk per 100 cc. of medium, this amount was selected as the basis for comparisons of the growth obtained from the same amount of milk heated for the various lengths of time given above.

The precision of this method is brought out by the fact that the probable error of the mean result of the control (with no milk included) is $\pm$0.00004 gm. on an average growth of 0.0031 gm., making the average growth seventy-seven times the probable error, while the probable error of the mean result of the experiments including 0.40 gm. of milk per 100 cc. of medium is $\pm$0.00025 gm. on an average growth of 0.0451 gm., making the average growth 180 times the probable error. The difference in growth between the control set (with no milk) and that in which the 0.4 gm. of milk was included is 168 times the probable error of the difference.

Comparison of the growth obtained in parallel tests with heated and unheated milk indicates a small destruction of the vitamin as the result of heating but not sufficient to permit of a measurement of the influence of the time of heating upon the amount destroyed.

While yeast grown in the foregoing medium is stimulated by the addition of milk up to 0.4 gm. per 100 cc., experiments reported by Fulmer and his co-workers (16, 21) since the foregoing experiments were begun make it appear doubtful that this medium is an adequate one in all

respects but vitamin B. These authors claim that such stimulation cannot be due to vitamin B, since they have developed a medium of known constituents which, when tested by the cell count method, did not seem to be improved by the addition of water-soluble B vitamin. Experiments analogous to those with the Williams medium were carried out on the Fulmer, Nelson, and Sherwood medium, which has the following composition per 100 cc.:

Grams	
0.188	NH_4Cl
0.100	$CaCl_2$
0.100	K_2HPO_4
0.040	$CaCO_3$
0.600	dextrin
10.000	sugar

Since the amount of $CaCO_3$ used was far in excess of its solubility, it was necessary to weigh this constituent out into each 100 cc. portion used as a test. The rest of the medium was made up in two 500 cc. portions (the $CaCl_2$ in one portion and the rest of the constituents in another) to avoid having to pipette off the insoluble calcium phosphate formed. Each of ten aliquot portions from the foregoing two solutions was then used as a test. Each aliquot portion in an Erlenmeyer flask, stoppered with cotton, was sterilized, cooled to 30°, seeded with yeast, and incubated for the same time and temperature as for the Williams medium. The yeast was made up for seeding as described above.

The results obtained in experiments with this medium could not be interpreted in terms of yeast growth because of the insoluble buffers used.

In experiments in which the yeast was burned after weighing in order to correct the control-weight for changes in the amount of insoluble mineral matter during the growth of the yeast, the results were not such as to indicate that this medium can be thus adapted to use with the gravimetric yeast method.

RAT METHOD

EXPERIMENTAL PROCEDURE

Rats, when twenty-eight to thirty days old, were divided into groups so selected that each animal on the diet containing the heated milk was checked by a brother or sister from the same litter and of approximately the same weight on the diet containing the unheated milk. For example, the following rats from the same litter were divided as follows:

Unheated Milk	Six-Hour Heated Milk	Twelve-Hour Heated Milk
No. 4595 ♂ (30 gms.)	No. 4593 ♂ (32 gms.)	No. 4594 ♂ (31 gms.)
No. 4599 ♀ (31 gms.)	No. 4598 ♀ (31 gms.)	No. 4597 ♀ (32 gms.)
	No. 4600 ♀ (29 gms.)	No. 4601 ♀ (29 gms.)

The diet used was as follows:

	Per Cent
Starch	50
Dry skimmed-milk powder	40
Purified butter fat	9
Sodium chloride	1

The skimmed-milk powder had the following composition:

	Per Cent
Moisture	3.0
Fat	0.3
Protein	33.3
Carbohydrate	56.3
Ash	7.2

The butter fat was prepared by melting at low temperature and then cooling. This caused most of the curd, salt, and water to separate out from the fat, which was then removed, melted at low temperature and filtered through filter paper. The butter fat was well mixed with the starch, and the salt and milk added. The whole was then well mixed in a mechanical mixer. The groups to be compared with the control group were fed the same diet except that the skimmed-milk powder had been heated at 100° C. for various lengths of time (six, twelve, twenty-four, and forty-eight hours respectively as given above in the yeast experiments).

The rat was adopted as the best experimental animal because it was best standardized by previous work. The ration here used was based on the following considerations: (1) It derives vitamin B entirely from one source and this is a material which is readily adaptable to experiments with heat treatment either wet or dry. (2) Forty per cent of the weight, or 33 per cent of the calories in the form of skimmed-milk powder, is approximately the minimal proportion for optimum growth as indicated by unpublished experiments performed in this laboratory by Miss F. L. MacLeod. (3) With skimmed-milk powder and butter fat furnished separately, the former can, if desired, be subjected to heating or other treatment or to modification of amount without affecting the adequacy of the supply of vitamin A in the butter fat. (4) This diet corresponds approximately on the calorie basis to Osborne and Mendel's whole milk-starch-fat diet which was found to be adequate. (5) Experiments by Sherman and Merrill have shown that when growth is retarded by further dilution of milk powder with starch, vitamin B is the first limiting factor. Hence this control diet can be considered analogous to the Williams

medium in the yeast experiments when 0.4 gm. of unheated milk per 100 cc. of medium were included.

The gain in body weight and the average food consumption of each animal were determined weekly for a period of eight weeks. (About one-third of the animals was kept on the experiment twelve weeks and corroborated previous and subsequent experience in the same laboratory, that any significant difference in growth under these conditions will be shown in the portion of the growth curve corresponding to the ages four to thirteen weeks.)

The food and water in each cage were replenished often enough so that the animals were always provided with food and water and therefore ate *ad libitum*. The food left and recovered on cleaning the cage subtracted from that offered the rats was taken as the amount of food consumed by the group.

RESULTS OF EXPERIMENTS WITH RATS

A comparison of the average gains and probable errors made during the eight weeks by the rats on the unheated and heated milk are given in the following tables. Each table represents the results obtained on the animals studied during a given season.

TABLE IV

COMPARISON OF GAINS AND PROBABLE ERRORS GIVEN SEPARATELY FOR ANIMALS STUDIED DURING EACH SEASON

Milk	No. of Rats	Average Gains (Grams)	Probable Error on the Gains (Grams)	Decrease in Gain (Grams)	Probable Error of Decreased Gain (Grams)
	First Season				
Unheated	11	125	4.0	.....	.....
Heated 6 hours at 100° C.	5	117	4.6	8	6.1
Heated 24 hours at 100° C.	7	79	2.1	46	4.5
Heated 48 hours at 100° C.	8	67	1.8	58	4.4
	Second Season				
Unheated	39	92	2.2	.....	.....
Heated 24 hours at 100° C.	14	49	1.7	43	2.7
Heated 48 hours at 100° C.	14	24	1.6	58	2.7
	Third Season				
Unheated	14	99	2.9	.....	.....
Heated 6 hours at 100° C.	22	79	2.2	20	3.6
Heated 12 hours at 100° C.	23	77	1.8	22	3.4

The difference in gains between those animals on unheated milk and those on diets containing milk heated twelve, twenty-four, and forty-eight hours is in each case greater than six times its probable error, while the difference corresponding to the two longer periods of heating varies from ten to twenty-one times its probable error. When the total of all animals used, on each modification of the diet, is compared with the total control animals used, the results are as shown in Table V. Here the differences corresponding to the periods of heating are respectively four, eight, twelve, and fifteen times the probable error of the difference, and therefore seem clearly significant.

TABLE V

COMPARISON OF GAINS AND PROBABLE ERRORS OF ALL ANIMALS USED ON EACH VARIATION OF THE DIET

Milk	No. of Rats	Average Gains (Grams)	Probable Error on Gains (Grams)	Decreased Gain (Grams)	Probable Error on Decreased Gain (Grams)
Unheated	64	100	2.0	.....	.....
Heated 6 hours at 100° C...	28	86	2.7	14	3.4
Heated 12 hours at 100° C...	23	77	1.8	23	2.7
Heated 24 hours at 100° C...	21	59	2.5	41	3.4
Heated 48 hours at 100° C...	22	40	3.3	60	3.9

When ten animals on each variation of the heat treatment were selected for comparison with ten on the control diet, each animal used as a test of the heat treatment being checked by a brother or sister from the same litter and as near the same weight as possible in the control set, the results are as shown in Table VI. On the basis of this comparison

TABLE VI

COMPARISON OF GAINS AND PROBABLE ERRORS OF TEN TYPICAL ANIMALS ON EACH MODIFICATION OF THE HEAT TREATMENT, WITH LITTER-CONTROLS ON THE UNHEATED MILK

Milk	No. of Rats	Average Gains (Grams)	Probable Error on Gains (Grams)	Decreased Gains (Grams)	Probable Error on Decreased Gains (Grams)
Unheated	10	99	3.6	.....	.....
Heated 6 hours at 100° C...	10	97	3.6	2	5.1
Unheated	10	97	3.6	.....	.....
Heated 12 hours at 100° C...	10	86	2.4	11	4.3
Unheated	10	100	2.2	.....	.....
Heated 24 hours at 100° C...	10	56	3.6	44	4.2
Unheated	10	116	2.2	.....	.....
Heated 48 hours at 100° C...	10	51	4.9	65	5.4

the differences corresponding to the twelve, twenty-four, and forty-eight-hour heated milk would be considered statistically significant, being respectively two and one-half, ten, and twelve times the probable error.

The difference in the gains of the rats whose diet contained the heated milk represents the percentage reduction of growth, but the percentage reduction of vitamin would probably be less because some is required for maintenance and only the surplus above the maintenance requirement would be available for growth and therefore measurable.

DISCUSSION OF FOOD CONSUMED

(*a*) The average grams of food consumed per rat, (*b*) the calories consumed per rat per gram of gain, and (*c*) the grams gained per thousand calories consumed, both by seasons, and for the total animals studied on each variation of the diet, are given in the following tables.

TABLE VII

Milk	Average Grams Food Eaten per Rat in the 56 Days of Experiment	Calories Eaten per Rat per Gram of Gain	Grams Gained per Rat per 1,000 Calories
	First Season		
Unheated	681	23.2	43.2
Heated 6 hours at 100° C.	606	22.0	45.4
Heated 24 hours at 100° C.	497	26.7	37.0
Heated 48 hours at 100° C.	441	28.0	30.4
	Second Season		
Unheated	619	28.6	34.9
Heated 24 hours at 100° C.	380	33.0	20.3
Heated 48 hours at 100° C.	372	66.0	15.1
	Third Season		
Unheated	611	26.2	38.4
Heated 6 hours at 100° C.	484	26.0	38.4
Heated 12 hours at 100° C.	501	27.7	36.1

The record for food consumption shows that the rats on the diets containing the heated milk ate fewer grams of food than did the control animals. However, although the animals on the diets containing the milk heated for twenty-four and forty-eight hours actually consumed fewer grams of food than did the control animals, they required more calories to produce one gram of gain, or gained fewer grams per calorie

consumed than did the control animals. This was due, of course, to the smaller gains made by those on the heated food.

The difference in the food consumption of the various animals cannot be stated with as great precision as can the body weights and the gains computed therefrom. The weekly weight can be stated with a precision of ±1 gm., whereas the food consumed during one week cannot be

TABLE VIII

Food Consumption of All Animals on Each Variation of the Diet

Milk	Average Grams Food Eaten per Rat	Calories per Rat per Gram of Gain	Grams Gained per Rat per 1,000 Calories Eaten
Unheated	537	27.1	36.9
Heated 6 hours at 100° C.	513	25.4	39.4
Heated 12 hours at 100° C.	501	27.6	36.1
Heated 24 hours at 100° C.	439	31.6	31.6
Heated 48 hours at 100° C.	406	41.3	23.1

calculated with this precision because the animals, particularly those on diets containing the heated milk, scattered their food about the cages. Although the cages in most cases were cleaned every day, and the scattered food recovered as completely as possible, the loss from this cause was considerable. Therefore the error on the values given for the food consumed is on the positive side and the actual consumption was less than would be indicated by these values.

SUMMARY AND CONCLUSIONS

The purpose of this work was to examine the availability of either or both of two methods for the study of such a problem as the heat destruction of vitamin B. The methods studied were (*a*) the yeast-growth method of Williams in its gravimetric form, supplemented by experiments with the medium proposed by Fulmer, Nelson, and Sherwood; (*b*) the rat-growth method extensively employed in the laboratories of Hopkins, of Osborne and Mendel, of McCollum, and elsewhere for the testing of the adequacy of experimental diets in general.

Over 300 quantitative determinations by the gravimetric yeast-growth method and over 150 quantitative studies of the growth of young rats have been completed. The data secured with both methods have been such as to permit of the discussion and interpretation of the results upon a quantitative basis.

The gravimetric yeast-growth method of Williams seems the most promising of the quick methods permitting easy control of conditions

throughout an experiment and relatively rapid and numerous repetitions by means of which to reduce the probable error of the mean result. The data obtained make it clear that this method as here described is capable of yielding consistent results, the coefficient of variability and probable error of which can be kept within limits quite satisfactory for the purposes of vitamin research as at present developed. The Williams method is, however, open to the objection that the increased growth of yeast which it measures may be due to the introduction of other substances favorable to yeast growth as well as the vitamin B. The medium devised by Fulmer, Nelson, and Sherwood to overcome this objection is not adapted to gravimetric use especially because of its employment of insoluble buffers. The counting of cells yields results too variable for satisfactory quantitative discussion.

The rat-growth method involves somewhat larger probable errors than the Williams yeast method, but can be interpreted in terms of the B vitamin with much greater certainty and is therefore the preferable method.

So far as is practicable each experimental animal should have a closely comparable control in the from of a twin of nearly the same initial size, so that a series of comparisons can be made between animals confidently regarded as directly and accurately comparable. The conclusion reached through such comparison of individual animals with litter-controls should then be verified by comparison of the general averages of groups so large as to insure that any individual peculiarities cannot appreciably affect the main result.

In the work here described comparisons are made on both these bases and furthermore by treating the work of each of three seasons as a separate series and comparing the means of each of the three series separately. All three methods of comparison lead to consistent results, thus establishing quite definitely the applicability of the rat-growth method to such studies of vitamin B as are here under consideration. The work as a whole indicates clearly that the rat-growth method is the best means as yet available for use in studies of the B vitamin.

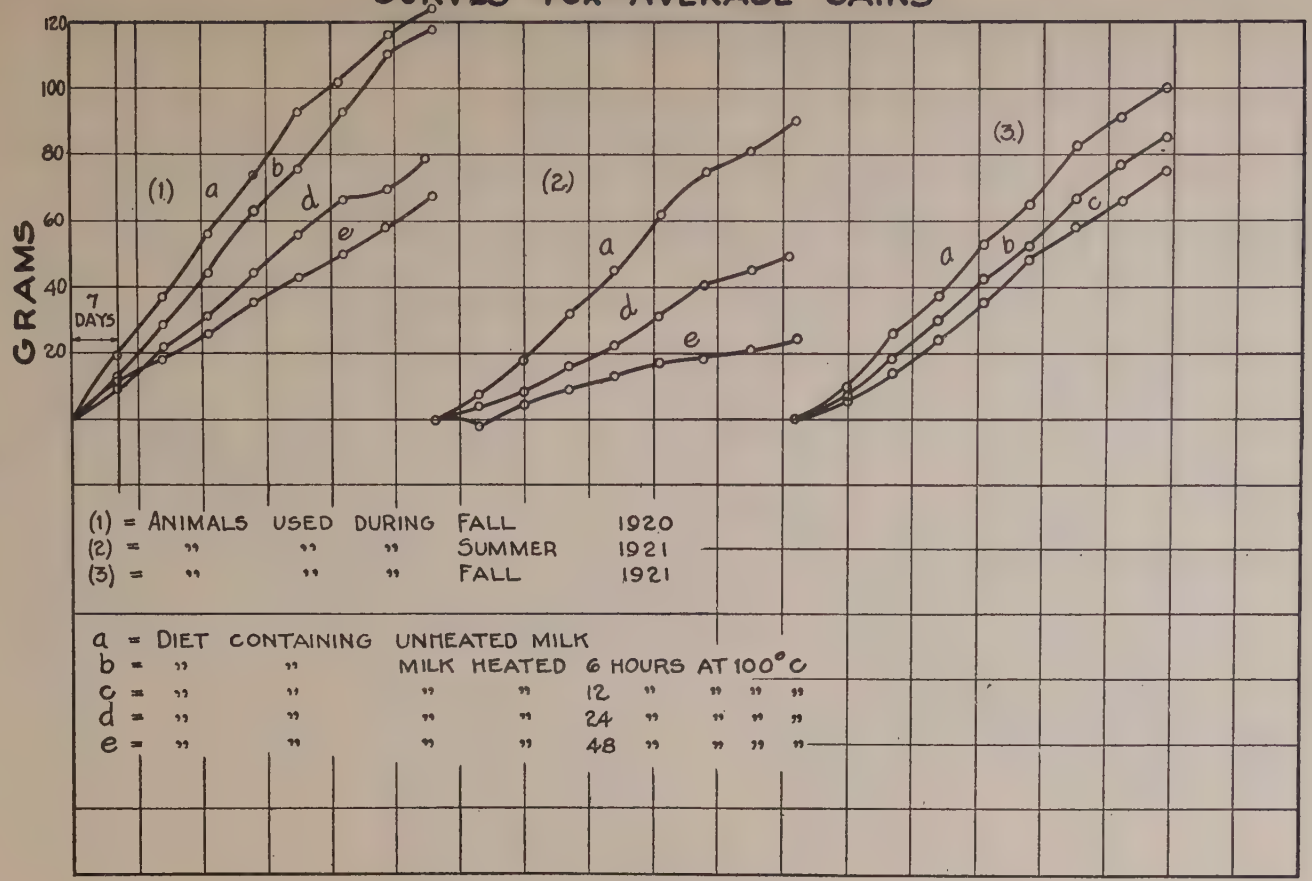
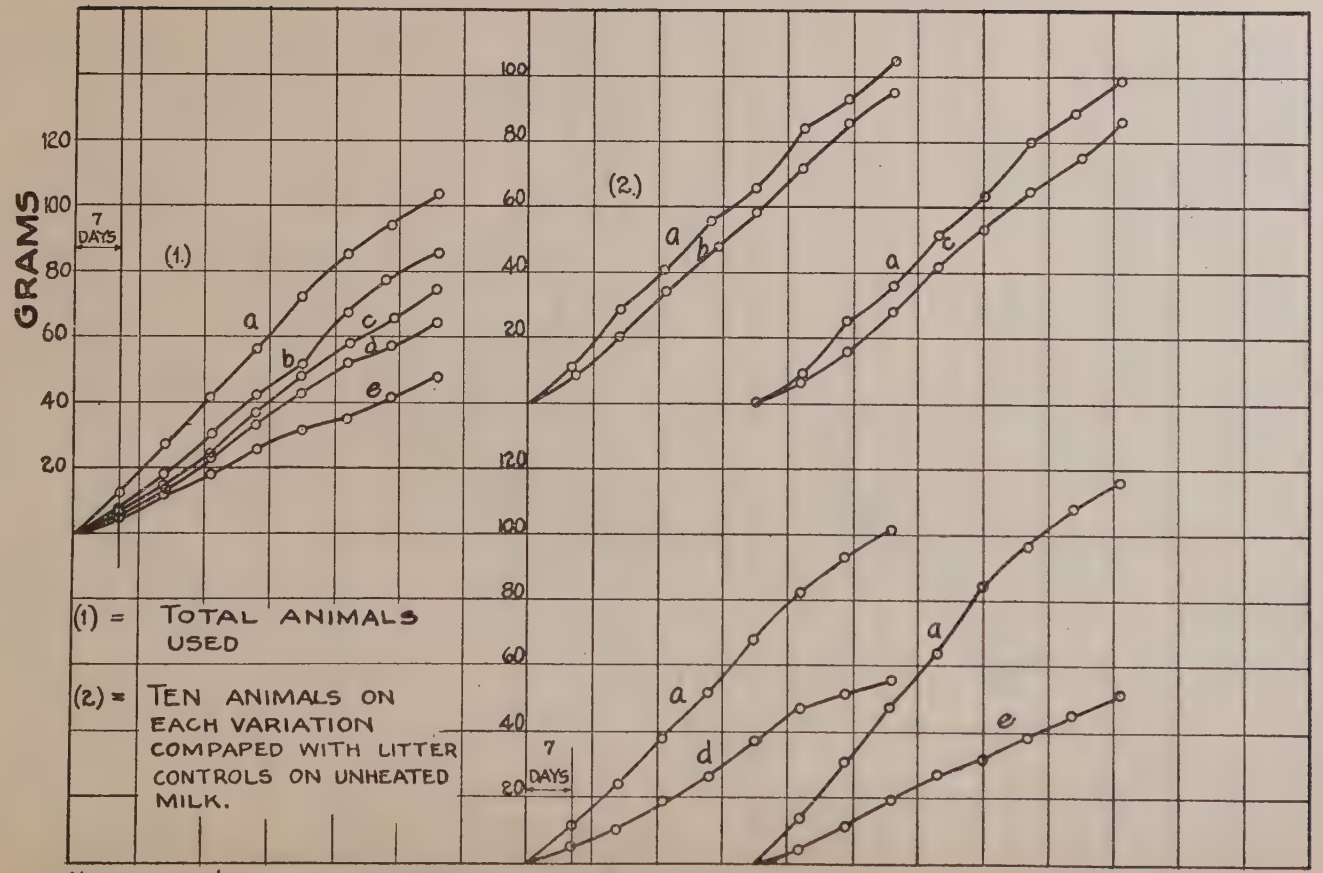

CURVES FOR AVERAGE GAINS
GRAMS
120
100
80
60
40
20
7 DAYS
(1)
(2)
(3)
a
b
c
d
e
(1) = ANIMALS USED DURING FALL 1920
(2) = " " " SUMMER 1921
(3) = " " " FALL 1921
a = DIET CONTAINING UNHEATED MILK
b = " " MILK HEATED 6 HOURS AT 100° C
c = " " " " 12 " " " "
d = " " " " 24 " " " "
e = " " " " 48 " " " "

CHART I

CURVES FOR AVERAGE GAINS



NOTE. a,b,c,d e, SAME AS CHARTS Nº 1, 3 AND 4.

CHART II

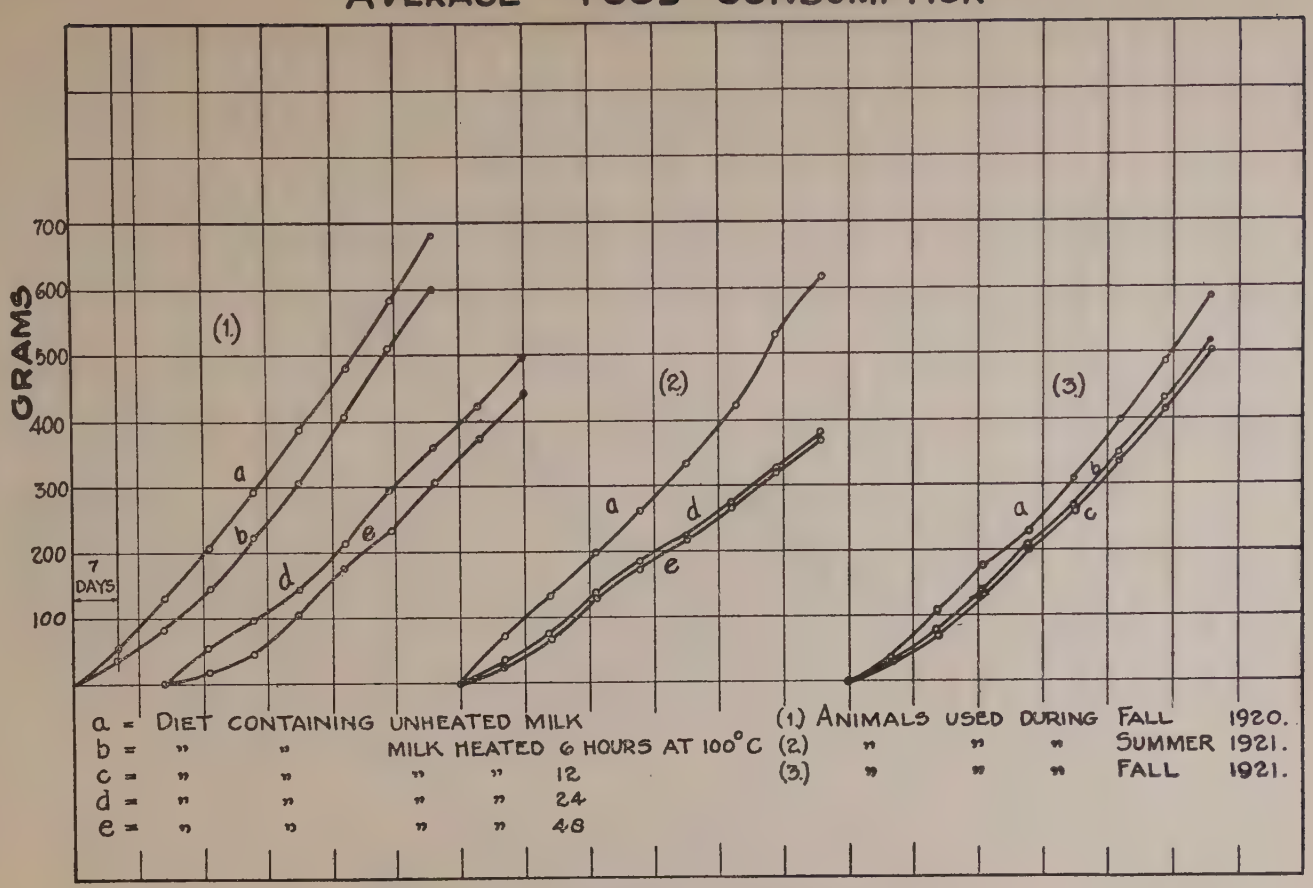

AVERAGE FOOD CONSUMPTION
GRAMS
700
600
500
400
300
200
100
7 DAYS
(1)
(2)
(3)
a
b
c
d
e
a = DIET CONTAINING UNHEATED MILK
b = " " MILK HEATED 6 HOURS AT 100° C
c = " " " " 12
d = " " " " 24
e = " " " " 48
(1) ANIMALS USED DURING FALL 1920.
(2) " " " SUMMER 1921.
(3) " " " FALL 1921.

CHART III

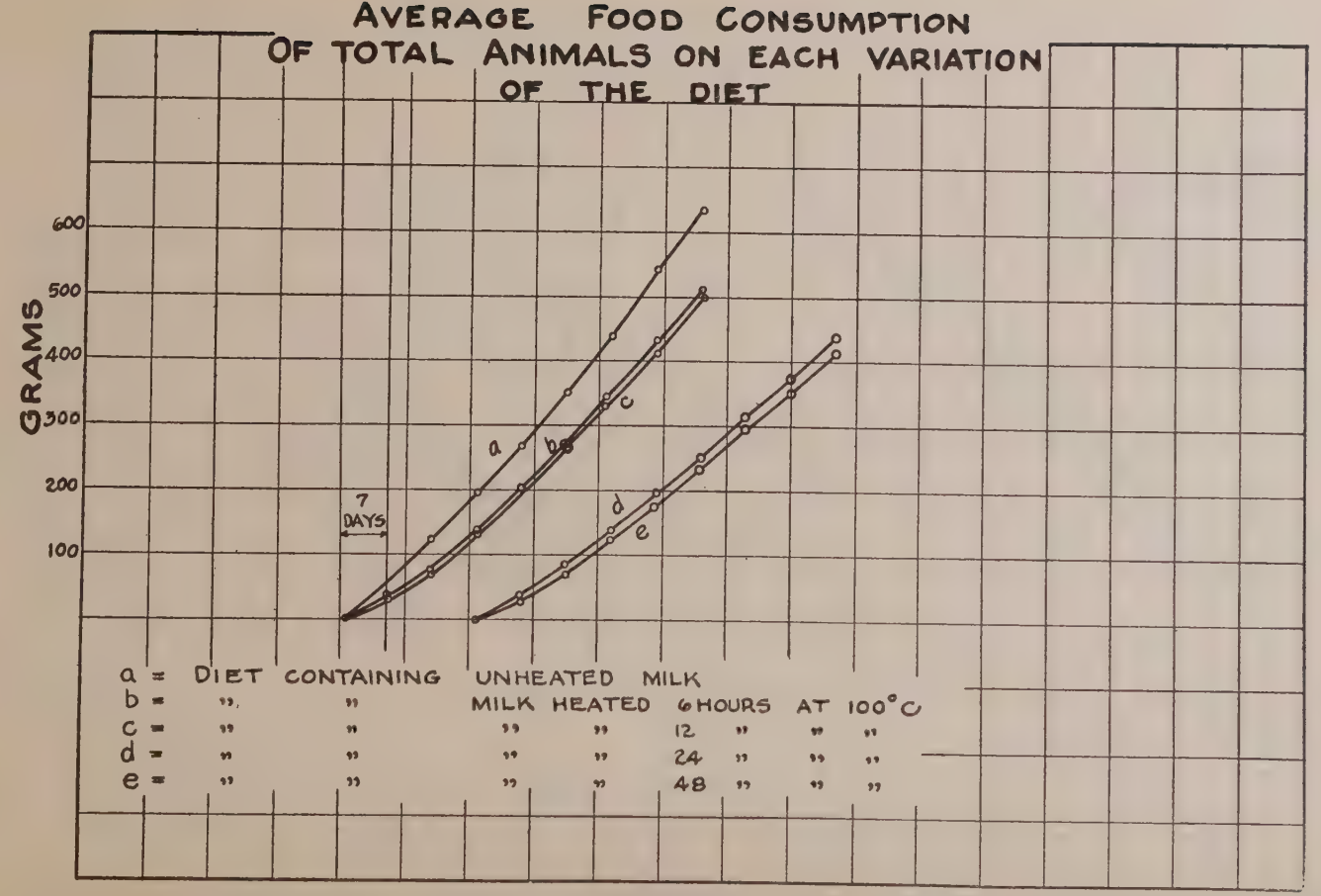

AVERAGE FOOD CONSUMPTION
OF TOTAL ANIMALS ON EACH VARIATION
OF THE DIET
GRAMS
600
500
400
300
200
100
7
DAYS
a
b
c
d
e
a = DIET CONTAINING UNHEATED MILK
b = " " MILK HEATED 6 HOURS AT 100°C
c = " " " " 12 " " "
d = " " " " 24 " " "
e = " " " " 48 " " "

CHART IV

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